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A rapid bioassay for bactericides against the catfish pathogens *Edwardsiella ictaluri* and *Flavobacterium columnare*

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Abstract

The most common bacterial diseases in pond-raised channel catfish *Ictalurus punctatus* (Rafinesque) are enteric septicemia of catfish and columnaris, caused by *Edwardsiella ictaluri* and *Flavobacterium columnare* respectively. Medicated feed containing antibiotics is one management approach that catfish producers use in the treatment of bacterial diseases. However, the future use of all types of medicated feed in catfish aquaculture is uncertain. To discover effective alternatives to antibiotics, a rapid 96-well microplate bioassay utilizing *E. ictaluri* and *F. columnare* to evaluate natural compounds and extracts was developed. In this bioassay, bacterial growth is determined by absorbance measurements of microplate wells after 24 h incubation and then confirmed by detecting cell viability after the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide with additional incubation for 24 h. The minimum inhibitory concentration , minimum bactericidal concentration and 50% inhibition concentration (IC50) are determined by graphing the absorbance data. The 24 h IC50 results of test compounds are compared with the 24 h IC50 results of the drug controls oxytetracycline and florfenicol. Among the antibiotics evaluated, doxycycline and tetracycline appear more effective against *E. ictaluri* and *F. columnare* than either drug control. This bioassay is rapid, reproducible and economical for evaluating a large number of compounds and extracts.

Keywords: bioassay, catfish, columnaris, *Edwardsiella ictaluri*, enteric septicemia, *Flavobacterium columnare*

Introduction

The two leading infectious diseases that cause the highest mortalities of farm-raised channel catfish *Ictalurus punctatus* (Rafinesque) are columnaris and enteric septicemia of catfish (ESC) (Hawke & Khoo 2004). Columnaris and ESC are caused by the Gram-negative, rod-shaped bacteria *Flavobacterium columnare* and *Edwardsiella ictaluri* respectively. Columnaris can occur as separate infections or as mixed infections with *E. ictaluri*, thereby making it difficult to determine the exact economic impact of columnaris disease on the catfish industry. Based upon direct and indirect losses of market-sized catfish attributed to ESC and columnaris in 2003 (USDA/APHIS 2003), these diseases may cost the $2.5 billion catfish industry well over $100 million annually.

The use of medicated feed is one management approach that catfish producers use in the treatment of ESC. Previously, the only antibiotic approved by the United States Food and Drug Administration (USFDA) as a feed additive to treat ESC was Romet-30® (sulfadimethoxine-ormetoprim). During 2005, Aquaflor® (florfenicol) was approved by the USFDA for use in commercial catfish aquaculture to manage ESC (USFDA/CVM 2005). Currently, there are no specific approvals by USFDA of antibiotics for treating columnaris in catfish.

Another management approach is the use of therapeutants such as potassium permanganate ($KMnO_4$) and copper sulphate pentahydrate ($CuSO_4 \cdot H_2O$) to treat columnaris, though neither one is specifically approved by the USFDA for catfish disease treatment (Plumb 1999). In fact, Plumb (1999) suggests that the application of $KMnO_4$ in combina-

tion with Terramycin® for fish (Pfizer Inc., Lee's Summit, MO, USA) (oxytetracycline)-medicated feed as the most effective manner in which to treat columnaris-infected channel catfish. However, these therapeutants have several drawbacks including the following: (1) they interact strongly with water quality; (2) they are highly phytotoxic and (3) they have broad-spectrum toxicity which requires careful application so that catfish are not killed (Boyd & Tucker 1998). The discovery of environmentally safe natural compounds that are selectively toxic towards *E. ictaluri* and *F. columnare* would benefit the catfish industry.

The future use of all types of medicated feed in catfish aquaculture is uncertain due to negative attributes. These negative attributes include the following: (1) potential development of antibiotic resistant strains of *E. ictaluri* and *F. columnare* from the use of antibiotics and (2) public concerns about the environmental impact from the use of antibiotic-laden feeds in agriculture.

In order to discover natural compounds as alternatives to currently used antibiotics and for use as bactericides (therapeutants) against *E. ictaluri* and *F. columnare*, we developed a rapid 96-well microplate bioassay to evaluate natural compounds and extracts (e.g., from plants). The bioassay measures cell viability by incorporation of the yellow dye 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into the protocol, and this bioassay is rapid, reproducible and economical for evaluating a large number of compounds and extracts.

Materials and methods

Bacterial strains and culturing

Cultures of *E. ictaluri* (strain #1039) and *F. columnare* (strain #1016) were obtained from Mr Tim Santucci (College of Veterinary Medicine, Mississippi State University, Stoneville, MS) and were grown in 3.8% Mueller–Hinton (MH) broth (pH 7.3) (Becton, Dickinson and Company, Sparks, MD, USA) supplemented with 3.9% sterile fetal calf serum (FCS) (Hardy Diagnostics, Santa Maria, CA, USA). The FCS was added to the MH broth after sterilization and cooling to 55 °C. Maintenance of stock cultures and culture purity checks were performed using 3.8% MH agar (Becton, Dickinson and Company) plates supplemented with 3.9% FCS for *F. columnare* and 3.9% sterile sheep blood (Hardy Diagnostics) for *E. ictaluri*.

For conducting the bioassay, both species were transferred from stock cultures to fresh 3.8% MH–FCS agar plates and grown at 29 °C for 3–5 days until the average diameter of the colonies was 0.1 and 0.2 cm for *E. ictaluri* and *F. columnare* respectively. Isolated colonies of each bacterial strain were aseptically transferred to 6 mL 3.8% MH–FCS broth in separate glass test tubes and vortexed 5–10 s until visible clumps of bacteria were disrupted. Appropriate volumes of cell concentrates were aseptically transferred to 30 mL of fresh 3.8% MH–FCS broth in 50 mL Nalgene glass media bottles to obtain 0.5 McFarland standard using a spectrophotometer (model UV-3101PC; Shimadzu, Kyoto, Japan). This procedure provided a culture suspension of actively growing cells containing 10^8 colony forming units (CFU) mL^{-1}, as confirmed by 3.8% MH–FCS agar plate counts.

Growth assay development

Sterile 96-well polystyrene microplates (type Costar; Corning, Corning, NY, USA) with flat-bottom wells were used. Initially, florfenicol (100% pure) and oxytetracycline hydrochloride (HCl) (95% pure) were evaluated in the development of the bioassay. In addition, cephalexin hydrate (100% pure), chloramphenicol (98% pure), doxycycline HCl (100% pure) and tetracycline HCl (100% pure) were evaluated. All antibiotics were obtained from Sigma-Aldrich (St Louis, MO, USA) and corrections for purity were made. These antibiotics were dissolved in either sterile, double-deionized water or 100% ethanol, and 10 μL of each serially diluted (10-fold) test compound was aseptically micropipetted into microplate wells (10 μL $well^{-1}$ and three replications for each dilution). Solutions made in water were filter sterilized (0.22 μm) before being added to microplate wells. Ethanol was allowed to completely evaporate before adding 200 μL of standardized bacterial culture (0.5 McFarland) per well while 190 μL of standardized bacterial culture was added to wells containing test compound dissolved in water.

Using a 96-well microplate template for each bacteria species, control wells (15 wells per microplate) did not contain test compound, and blank wells (5 wells per microplate) did not contain test compound or test bacteria. Drug controls of florfenicol and oxytetracycline HCl were included for each bacterial strain at the same concentrations as the test compounds. Compensations for the amount of active ingredient in the test compound (e.g., oxytetracycline in oxytetracycline HCl) were made. Final concentra-

tions of test compounds in the microplate wells were 0.01, 0.1, 1.0, 10.0, 100.0 and 1000.0 μM. Microplates were incubated at 29 °C for 24 h. A Packard model SpectraCount microplate photometer (Packard Instrument Company, Meriden, CT, USA) was used to measure the absorbance (630 nm) of the wells after microplate shaking (auto mixing of 7 s) at time 0 and 24 h. Each experiment was repeated.

MTT assay

The MTT assay was performed in a similar manner as described by Mshana, Tadesse, Abate and Miörner (1998), but significant modifications were made. A 0.22 μm syringe-filter unit (Millex GP, Millipore Corporation, Bedford, MA, USA) was used to filter sterilize MTT (Sigma-Aldrich) dissolved in phosphate-buffered saline (pH 7.2) (5 mg mL^{-1}). After 24 h, absorbance measurements were obtained and 40 μL of culture material from each microplate well used in the growth assay were aseptically micropipetted to a corresponding well in another sterile 96-well polystyrene microplate containing 10 μL of MTT previously micropipetted into each well. Culture material was transferred to wells of a new microplate as the well volume capacity of these microplates was limited to 250 μL. Each microplate was incubated for 4 h at 29 °C and then 50 μL of lysing buffer [20% sodium dodecyl sulfate in 50% *N,N*-dimethylformamide (pH 4.7)] was added to each well. Microplates were incubated for an additional 20 h and absorbance was measured (no mixing) with a Packard model SpectraCount microplate photometer (Packard Instrument Company) at 570 nm. Microplate wells that contained 3.8% MH–FCS broth, MTT and lysing buffer were used as blanks.

Before initiation of the MTT assay, 5 μL of culture material were aseptically transferred using a micropipetter from each growth assay microplate well onto 3.8% MH–FCS agar plates. Agar plates were inverted and incubated at 29 °C for 24 h for *F. columnare* and 5 days for *E. ictaluri*. Plates were observed for growth and the minimum bactericidal concentration (MBC) was determined to be the lowest concentration at which no growth was observed on the agar.

Comparison of growth assay and MTT assay

Three separate bioassays (growth and MTT assays) for *E. ictaluri* and *F. columnare* with different concentrations (0.01, 0.1, 0.5, 1.0, 10.0, 100.0 and 1000.0 μM) of florfenicol and oxytetracycline HCl were performed. After 24 h of incubation, absorbance measurements of each microplate well for the growth assay and the MTT assay were paired and analysed using correlation analyses to determine the Pearson correlation coefficient (r). For each experiment, the 24 h 50% inhibition concentration (IC50) values for florfenicol and oxytetracycline were determined using growth assay and MTT assay results. The statistical significance of the growth assay 24 h IC50 values compared with those obtained from the MTT assay was determined using a paired t-test (Microsoft Excel 97).

Incubation period for growth assay

As *E. ictaluri* grows more slowly than *F. columnare*, another study was performed to determine if the incubation period for the growth assay of *E. ictaluri* should be extended from 24 to 48 h. Duplicate microplates were set up for *E. ictaluri* in the same manner as discussed for development of the growth and MTT assays. Florfenicol and oxytetracycline HCl were used as the test compounds. One microplate was incubated for 24 h and the other for 48 h before initiation of the protocol for the MTT assay. The experiment was repeated. Both 24 and 48 h IC50 values were determined from the absorbance measurements obtained from each assay.

Reproducibility studies

In order to assess the reproducibility of the bioassay, cephalexin hydrate, chloramphenicol, doxycycline HCl and tetracycline HCl were evaluated in addition to florfenicol and oxytetracycline HCl. Each antibiotic was evaluated at several concentrations (0.01, 0.1, 1.0, 10.0, 100.0 and 1000.0 μM) and each bioassay was repeated. Drug controls (florfenicol and oxytetracycline HCl) were included to help confirm reliability of assay results for the other antibiotics evaluated.

Data analyses of growth and MTT assays

The means and standard deviations of absorbance measurements from the growth and MTT assays were calculated and graphed to help determine the minimum inhibitory concentration (MIC) and 24 h IC50 values for each test compound. The MTT assay results were used to determine cell viability and to confirm 24 h IC50 results obtained from the growth

assay. The 24 h IC50 values were calculated using the following formula:

$$\%\ \text{growth (or}\ \%\ \text{cell viability for MTT assay)} = 100(\text{AD} - \text{BD divided by PCD} - \text{BD})$$

where AD is the absorbance difference which is the average absorbance measurement of test compound microplate wells ($n = 3$) at a particular concentration (time 0) minus the absorbance measurement at 24 h; BD the blank difference which is the average absorbance measurement of blank microplate wells ($n = 5$) at time 0 minus the absorbance measurement at 24 h; PCD the positive control difference which is the average absorbance measurement of control microplate wells ($n = 15$) at time 0 minus the absorbance measurement at 24 h.

The percentage values calculated from the above formula were then graphed against logarithmic concentration values to help determine the 24 h IC50. The coefficient of variation (%) for 24 h IC50 values ($n = 2$) of each test compound as determined from the growth assay and the MTT assay was calculated to help validate the reproducibility of the bioassay.

To help interpret the results of the effectiveness of other compounds (e.g., antibiotics) compared with the drug controls florfenicol and oxytetracycline against *E. ictaluri* and *F. columnare*, a relative-to-drug-control florfenicol (RDCF) value and a relative-to-drug-control oxytetracycline (RDCO) value were determined. The MIC and IC50 results for each antibiotic tested were divided by the respective MIC and IC50 results obtained for florfenicol and for oxytetracycline to determine the RDCF and RDCO values. Those RDCF and RDCO values close to '1.0' indicate greater effectiveness of the compound against *E. ictaluri* and *F. columnare*.

Results

Based upon the observations of control growth curves, the bacterial strains grew logarithmically in the microplate wells. Therefore, the effect of florfenicol and oxytetracyline on the growth of *E. ictaluri* and *F. columnare* were easily determined from the graphed absorbance measurements obtained from the growth assay. The MIC values were determined to be the concentration that inhibited growth, but did not overlap with the standard deviation of the MBC concentration (Fig. 1a–d). The MBC values were initially determined to be the concentration that completely inhibited growth (i.e., descending curve). The MBC values were confirmed by observations of the inoculated 3.8% MH–FCS agar plates.

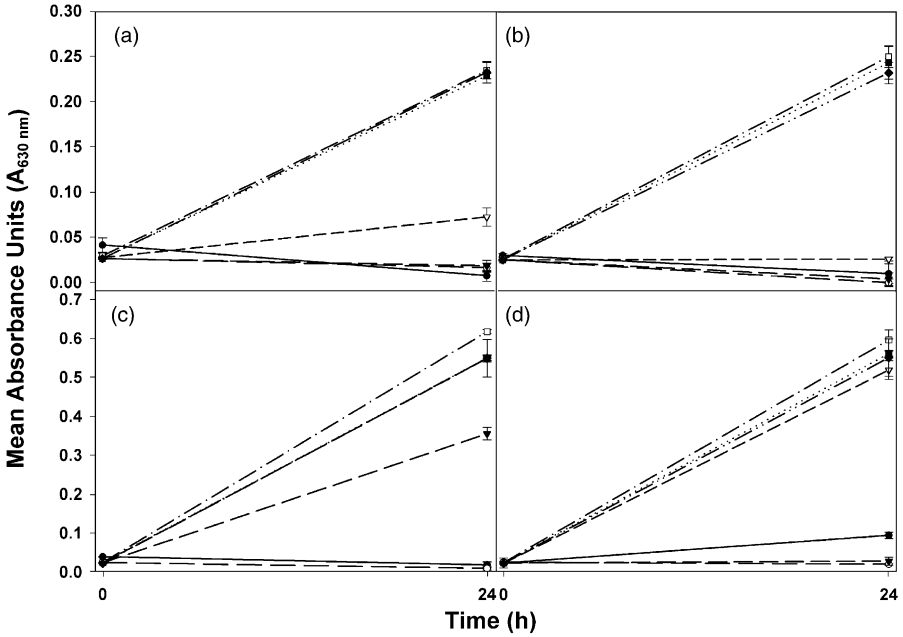

Figure 1 Effect of different concentrations of florfenicol and oxytetracyline on the growth of *Edwardsiella ictaluri* (a and b respectively) and *Flavobacterium columnare* (c and d respectively) cultured in a 96-well microplate ($n = 3$). The vertical bars represent the standard deviation of the mean. Control,-·-◆-·-; 0.01 μM,-·-⊡-·-; 0.1 μM, ·····■·····; 1.0 μM, ·····▽·····; 10.0 μM, ·····▼·····; 100.0 μM, - -⊖- -; 1000.0 μM, —●—.

The MTT assay was utilized to determine cell viability and to confirm MIC and 24 h IC50 results from the growth assay. An example of the graphed results obtained from one set of experiments is provided in Figs 1a–d, 2a–d, 3a, b and 4a, b. For *E. ictaluri*, the MIC of florfenicol was determined to be 1.0 μM or 0.358 mg L^{-1} (Fig. 1a), and the MBC of florfenicol was 1000.0 μM or 358 mg L^{-1} based upon visual inspection of the 3.8% MH–FCS agar plates. The MTT assay results (Fig. 2a) were used to confirm the MIC results obtained from the growth assay. For *E. ictaluri*, the 24 h IC50 value of florfenicol based upon growth assay results was 0.425 μM (0.152 mg L^{-1}) (Fig. 3a), whereas cell viability results revealed a 24 h IC50 value of 0.150 μM (0.054 mg L^{-1}) (Fig. 3b).

The MIC of oxytetracycline HCl for *E. ictaluri* was determined to be 1.0 μM or 0.497 mg L^{-1} (0.462 mg L^{-1} oxytetracycline as the active ingredient) (Figs 1b and 2b), and the MBC was 1000.0 μM or 497 mg L^{-1} (462 mg L^{-1} oxytetracycline as the active ingredient) based upon plate observation results. The 24 h IC50 value of oxytetracycline HCl based upon growth assay results was 0.315 μM (0.157 mg L^{-1}) or 0.146 mg L^{-1} oxytetracycline for *E. ictaluri* (Fig. 3a), whereas cell viability results revealed a 24 h IC50 value of 0.225 μM (0.112 mg L^{-1}) or 0.104 mg L^{-1} oxytetracycline (Fig. 3b).

The MIC of florfenicol for *F. columnare* was determined to be 10.0 μM or 3.58 mg L^{-1} (Figs 1c and 2c) while the MBC was >1000.0 μM or >358 mg L^{-1} based upon plating results. The 24 h IC50 value of florfenicol based upon growth assay results was 16.0 μM (5.73 mg L^{-1}) for *F. columnare* (Fig. 4a). Cell viability results revealed a 24 h IC50 value of 25.0 μM (8.96 mg L^{-1}) (Fig. 4b).

The MIC of oxytetracycline HCl for *F. columnare* was determined to be 10.0 μM or 4.97 mg L^{-1} (4.62 mg L^{-1} oxytetracycline as the active ingredient) (Figs 1d and 2d) and the MBC was 1000.0 μM or 497 mg L^{-1} (462 mg L^{-1} oxytetracycline). The high concentration of oxytetracycline HCl (1000.0 μM) may have contributed to the slightly higher absorbance measurements at 24 h compared with the absorbance measurements at lower concentrations (Fig. 1d). The 24 h IC50 value of oxytetracycline HCl based upon growth assay results was 3.0 μM (1.49 mg L^{-1}) (1.57 mg L^{-1} oxytetracycline) for *F. columnare* (Fig. 4a), whereas cell viability results revealed a 24 h IC50 value of 3.4 μM (1.69 mg L^{-1}) (Fig. 4b). The highest concentration of oxytetracy-

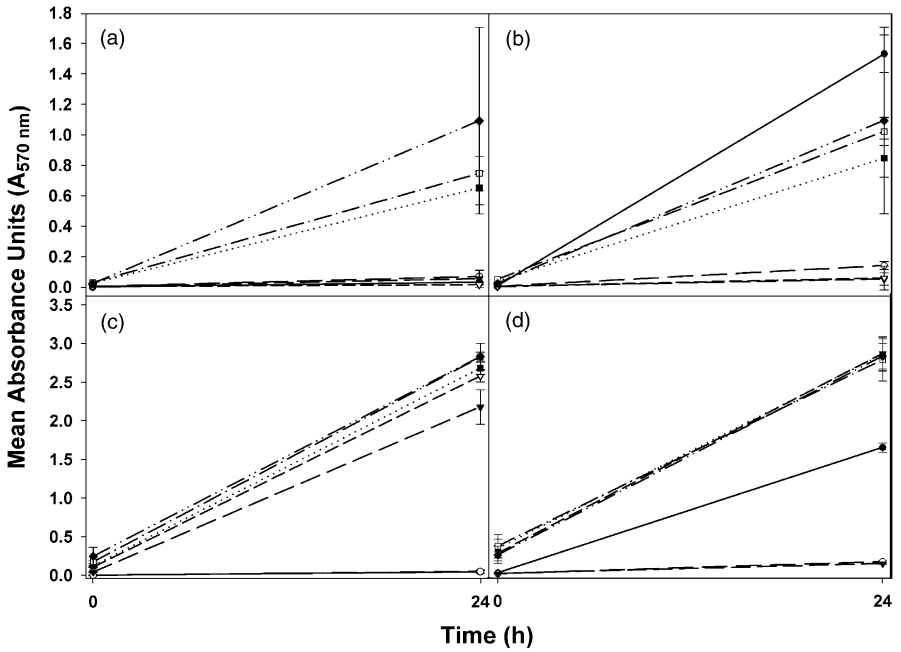

Figure 2 Effect of different concentrations of florfenicol and oxytetracyline on the metabolic activity of *Edwardsiella ictaluri* (a and b respectively) and *Flavobacterium columnare* (c and d respectively) cultured in a 96-well microplate ($n = 3$). The vertical bars represent the standard deviation of the mean. Control, ---◆---; 0.01 μM, ---□---; 0.1 μM, ·····■·····; 1.0 μM, ···▽···; 10.0 μM, ···▼···; 100.0 μM, - -⊖- -; 1000.0 μM, —●—.

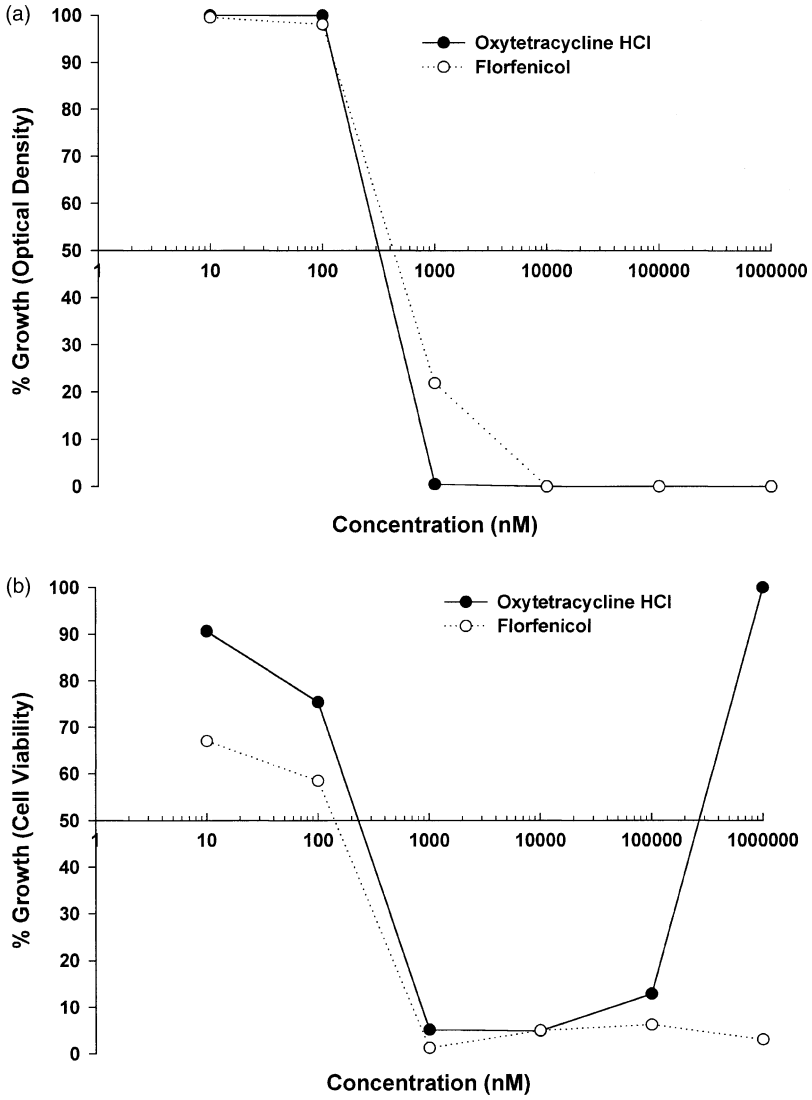

Figure 3 Determination of 24 h 50% inhibition concentration of florfenicol and oxytetracycline HCl for *Edwardsiella ictaluri* based upon absorbance measurements from the growth assay (a) and from the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (b).

cline HCl tested (1000.0 μM) appeared to have reacted with the MTT to help form formazan and provide much higher and 'false' absorbance readings concerning the measurement of cell viability (see Fig. 2b and d).

The Pearson correlation coefficients (r) that were calculated to compare the absorbance measurements from the growth assay to those obtained from the cell viability (MTT) assay for each microorganism indicated a strong correlation (graphs not shown). For *E. ictaluri*, correlation was high between growth assay and MTT assay results ($r = 0.92$, $P = 0.0001$, $n = 111$) and also for *F. columnare* ($r = 0.92$, $P = 0.0001$, $n = 123$) (additional microplate wells were used when setting up one replicate experiment). Comparison with the means (paired t-test) of the 24 h IC50 values for florfenicol and for oxytetracycline when tested against each organism indicated that the results obtained from the growth assay were not significantly different ($P \geq 0.05$) from those results obtained from the MTT assay.

Results for the study to help determine the incubation period, 24 or 48 h, for *E. ictaluri* when conducting the bioassay revealed that the growth assay 48 h IC50 values for florfenicol and oxytetracycline were much higher than the 24 h IC50 values. For example,

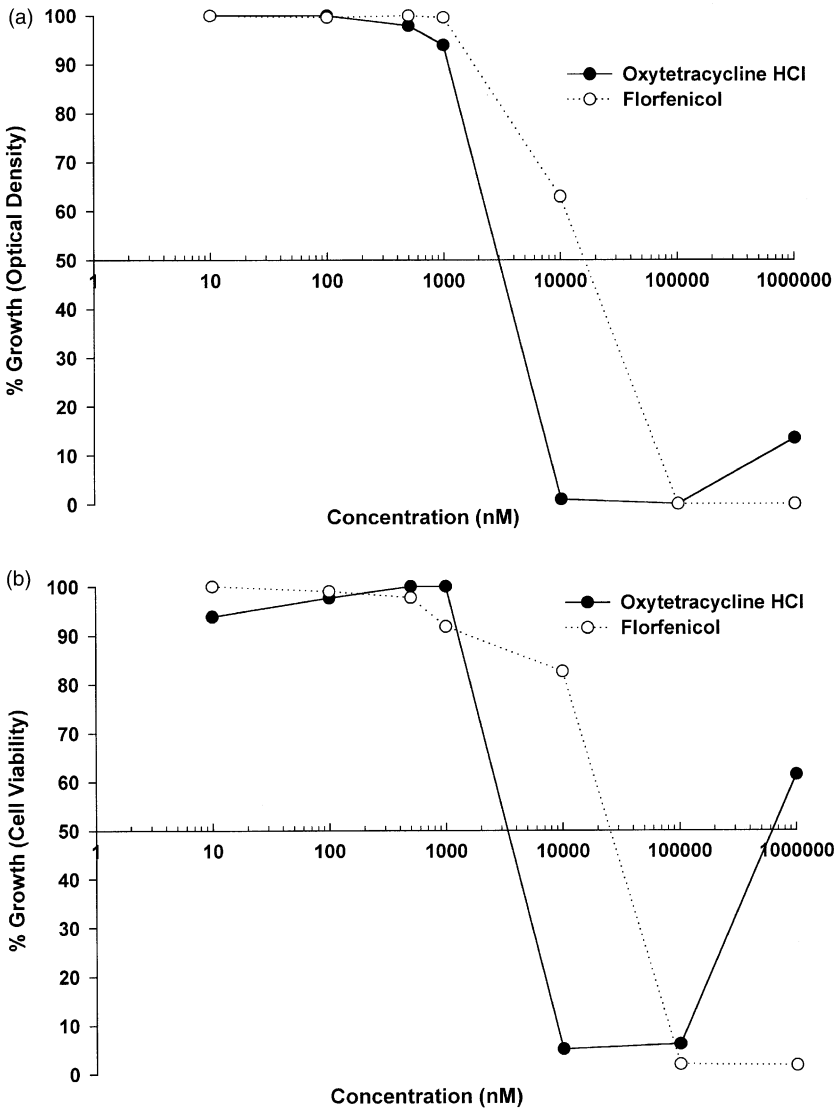

Figure 4 Determination of 24 h 50% inhibition concentration of florfenicol and oxytetracycline HCl for *Flavobacterium columnare* based upon absorbance measurements from the growth assay (a) and from the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (b).

the mean 48 h IC50 values for florenicol and oxytetracycline were 35.0 ± 12.54 and 1.86 ± 0.92, respectively, compared with mean 24 h IC50 values of 0.43 ± 0.15 and 0.29 ± 0.15 respectively. However, the MTT assay 48 h IC50 values increased much less compared with the 24 h IC50 values. For example, the mean 48 h IC50 values for florfenicol and oxytetracycline were 0.3 ± 0.11 and 0.28 ± 0.14, respectively, compared with mean 24 h IC50 values of 0.15 ± 0.05 and 0.21 ± 0.1 respectively.

For the reproducibility studies, results of mean MIC, MBC and 24 h IC50 values with standard deviations for florfenicol, oxytetracycline and the other antibiotics evaluated in this study are provided in Tables 1 and 2. The coefficients of variation provided in these tables ranged from 3.5% to 19.2%. The RDCF and RDCO values for MIC and 24 h IC50 of the antibiotics evaluated towards each microorganism are listed in Table 3.

Discussion

As first described by Mosmann (1983), the tetrazolium salt MTT has been used to assess cell viability in various bioassays (Peck 1985; Denizot & Lang

Table 1 MIC, MBC and 24 h IC50 values of compounds evaluated for toxicity towards *Edwardsiella ictaluri* using the bioassay

Compound*	MIC†‡	MBC†	24 h IC50†‡	CV§
Cephalexin	9.5 ± 0 (3.3)	>950 ± 0 (>330)	4.04 ± 0.34 (1.402)	8.4
Chloramphenicol	1.0 ± 0 (0.3)	1000 ± 0 (323)	1.85 ± 0.21 (0.598)	11.4
Doxycycline	0.9 ± 0 (0.4)	92 ± 0 (44)	0.36 ± 0.02 (0.171)	5.6
Florfenicol	1.0 ± 0 (0.4)	1000 ± 0 (358)	0.49 ± 0.09 (0.174)	18.4
Oxytetracycline	0.9 ± 0 (0.5)	930 ± 0 (462)	0.26 ± 0.05 (0.129)	19.2
Tetracycline	0.9 ± 0 (0.4)	924 ± 0 (444)	0.34 ± 0.04 (0.165)	11.8

*Results are reported as the amount of active ingredient contained in the test compound.
†Mean ± standard deviation from two independent experiments. Values are expressed in micromolar and values in parentheses are means expressed in milligram per litre.
‡Results are based upon growth assay with confirmation by MTT assay.
§Coefficient of variation as a percentage for 24 h IC50.
MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; IC50, 50% inhibition concentration; MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

Table 2 MIC, MBC and 24 h IC50 values of compounds evaluated for toxicity towards *Flavobacterium columnare* using the bioassay

Compound*	MIC†‡	MBC†	24-h IC50†‡	CV§
Cephalexin	9.5 ± 0 (3.3)	>950 ± 0 (>330)	30.9 ± 3.36 (10.726)	10.9
Chloramphenicol	10.0 ± 0 (0.3)	>1000 ± 0 (>323)	23.0 ± 1.41 (7.431)	6.1
Doxycycline	0.09 ± 0 (0.04)	92 ± 0 (44)	0.24 ± 0.04 (0.12)	16.7
Florfenicol	10.0 ± 0 (3.58)	>1000 ± 0 (>358)	15.5 ± 0.71 (5.55)	4.6
Oxytetracycline	9.3 ± 0 (4.62)	930 ± 0 (462)	2.86 ± 0.10 (1.42)	3.5
Tetracycline	0.9 ± 0 (0.44)	924 ± 0 (444)	1.69 ± 0.10 (0.81)	5.9

*Results are reported as the amount of active ingredient contained in the test compound.
†Mean ± standard deviation from two independent experiments. Values are expressed in micromolar and values in parentheses are means expressed in milligram per litre.
‡Results are based upon growth assay with confirmation by MTT assay.
§Coefficient of variation as a percentage for 24 h IC50.
MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; IC50, 50% inhibition concentration; MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

Table 3 The relative-to-drug-control values of compounds evaluated for toxicity towards *Edwardsiella ictaluri* and *Flavobacterium columnare* using the bioassay

	E. ictaluri				*F. columnare*			
	MIC		24 h IC50		MIC		24 h IC50	
Compound*	RDCF†	RDCO†	RDCF	RDCO	RDCF	RDCO	RDCF	RDCO
Cephalexin	9.5	10.2	43.6	82.2	1.0	1.0	2.0	10.8
Chloramphenicol	1.0	1.1	3.8	7.1	1.0	1.1	1.5	8.0
Doxycycline	0.9	1.0	0.7	1.4	0.01	0.01	0.02	0.08
Tetracycline	0.9	1.0	0.7	1.3	0.1	0.1	0.1	0.6

*Values are based upon the amount of active ingredient contained in the test compound.
†Values are the MIC and IC50 results in μM for each antibiotic divided by the MIC and IC50 results in μM for florfenicol and for oxytetracycline respectively.
MIC, minimum inhibitory concentration; IC50, 50% inhibition concentration; RDCF, relative-to-drug-control florfenicol; RDCO, relative-to-drug-control oxytetracycline.

1986; Hansen, Nielsen & Berg 1989; Stevens & Olsen 1993; Gomez-Flores, Gupta, Tamez-Guerra & Mehta 1995; Botsford 1997; Mshana *et al.* 1998; Schaller, Sun, Yang, Somoskovi & Zhang 2002). The process involves the conversion of the yellow dye MTT by mitochondrial dehydrogenase enzymes to produce

insoluble, purple formazan crystals which are then solubilized and measured spectrophotometrically. Although the procedure described by Mosmann (1983) utilized eukaryotic cells, Gomez-Flores *et al.* (1995) and Mshana *et al.* (1998) developed tests using *Mycobacterium* spp. Botsford (1997) also developed a colorimetric test utilizing MTT to determine the toxicity of compounds toward *Rhizobium melilotii*.

In the development of our bioassay, the MTT assay was used to confirm results obtained from the growth assay. As mentioned previously, correlations between cell turbidity and cell viability measurements for *E. ictaluri* and *F. columnare* were strong. However, the MTT assay does not account for those cells that have reduced metabolic activity but do retain sufficient ability to form CFU. In addition, some compounds at high concentrations can interact with MTT (e.g., oxytetracycline HCl) (Figs 3b and 4b) and lead to some difficulty in interpreting the graphed data. Therefore, the growth assay absorbance measurements were preferred for calculating MIC and 24 h IC50 results, and bioassay results were based upon the growth assay results. Also, the MTT assay could not be used solely to confirm MBC results. For example, compounds such as oxytetracycline HCl form a deep purple colour at high concentrations (e.g., 1000.0 μM as indicated in Figs 2b and 3b) and incorrectly indicate cell viability. Therefore, before initiation of the MTT assay, 5 μL of culture material should be removed from those growth assay microplate wells indicative of MBC (determined from the growth assay graphs) and plated onto 3.8% MH–FCS agar plates.

The inoculum level of 10^8 CFU mL^{-1} for each bacterial species was chosen for setting up the bioassay based upon the CFU used in previous studies and to help provide sufficient CFU to help produce measurable MTT reduction as previous studies showed that the degree of MTT reduction correlates with the number of bacteria (Gomez-Flores *et al.* 1995). In our study, this level of CFU was found to be adequate for the determination of compound toxicity in a 24 h period when using the MTT assay. The growth assay should be incubated for only 24 h as there is a large difference between 48 h IC50 values from the growth assay compared with the MTT assay (Table 2). The much larger increase in the growth assay 48 h IC50 values compared with the MTT assay 48 h IC50 values suggests that there is a larger proportion of dead or dying bacterial cells after 48 h of incubation. The dramatic increase in the absorbance readings at 48 h were attributed to the increased presence of less metabolically active cells.

Doxycycline, oxytetracycline and tetracycline had the lowest MIC values for *E. ictaluri* while doxycycline had the lowest MIC for *F. columnare* (Tables 1 and 2 respectively). Based on the MIC and 24 h IC50 results provided in Table 1, doxycycline and tetracycline appear to be slightly more effective than florfenicol and similar in effectiveness to oxytetracycline against *E. ictaluri* (Table 3). Doxycycline was found to have the lowest MBC (92.4 μM) towards *E. ictaluri*. In addition, doxycycline was found to be much more effective than florfenicol or oxytetracycline against *F. columnare* based on MIC, MBC and 24 h IC50 results (Table 2). For the two drug controls used in the bioassay, florfenicol was more effective in killing *E. ictaluri* than *F. columnare* based upon 24 h IC50 values (Tables 1 and 2). Oxytetracycline was also more effective against *E. ictaluri* than against *F. columnare*, and oxytetracycline was more effective than florfenicol in killing *E. ictaluri* and *F. columnare* based upon 24 h IC50 values (Tables 1 and 2 respectively). The coefficients of variation listed in Tables 1 and 2 are considered to be acceptably low as these values include within-assay variation and variations in CFU at the initiation of each separate experiment.

Relative to the drug controls, doxycycline appears to be more effective against *F. columnare* with RDCF and RDCO values for MIC and 24 h IC50 well below 1.0 (Table 3). Teteracycline is also more effective against *F. columnare* than either florfenicol or oxytetracycline.

As mentioned previously, the use of therapeutants to combat ESC and columnaris in catfish aquaculture is an alternative management approach rather than using medicated feed. However, therapeutic chemicals such as $KMnO_4$ and $CuSO_4 \cdot H_2O$ have been used less commonly to manage ESC than columnaris. Chloramine-T, diquat and hydrogen peroxide have also been investigated as bath treatments for columnaris disease in channel catfish, and diquat was found to be the most effective in reducing the mortality of acutely infected catfish (Thomas-Jinu & Goodwin 2004). However, diquat is labelled for aquatic use as an herbicide only and may be too expensive for use in large catfish production ponds. There are currently no bath or oral treatments approved by the USFDA for the treatment of columnaris disease in pond-raised channel catfish.

Plant extracts have been found to contain antimicrobial compounds effective against human pathogenic bacteria resistant to antibiotics (Nascimento, Locatelli, Freitas & Silva 2000) including *Staphylococcus aureus* (Pérez & Anesini 1994; Mansouri 1999). In

addition, antibacterials isolated from plants have been found to be effective against dental pathogenic bacteria (Tichy & Novak 1998). Research to discover and develop natural compounds (not antibiotics) for use as therapeutants to manage ESC and columnaris in catfish aquaculture has not been conducted previously. We plan to use this rapid bioassay to evaluate a large number of crude extracts from plants and other organisms (e.g., marine algae) to help discover active compounds that might be useful as therapeutants to manage ESC and columnaris in catfish production ponds.

In summary, this bioassay consists of two parts: the growth assay and the MTT assay (used to confirm growth assay results). The bioassay is rapid, reproducible and economical for screening large numbers of natural compounds and crude extracts to discover useful alternatives to antibiotics in managing ESC and columnaris in channel catfish aquaculture.

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